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13. ABSTRACT (Maximum 200 Words) AIB1 is a member of a coactivator family that potentiates the transcriptional activity of nuclear hormone receptors. The AIB1 gene is amplified in certain breast and ovarian cancers. AIB1 amplification is preferentially found in ER- and progesterone receptor-positive breast cancers. These findings suggest that AIB1 plays a causative role in breast cancer development. Our lab recently identified AIB1 as a target of the MAPK signaling pathway (Font de Mora and Brown, Mol Cell Biol 20:5041, 2000). This signaling pathway is triggered by growth factors of the insulin-like growth factor (IGF) and epidermal growth factor (EGF) family. These growth factors and their receptors have also been implicated in the development and progression of breast tumors. Based on these findings, we propose that the phosphorylation of AIB1 by MAPK may represent part of the molecular mechanism that integrates signals from steroid hormones and growth factors. Furthermore, we hypothesize that AIB1 phosphorylation may contribute to the role that AIB1 plays in the development of breast cancer. In order to identify the sites of AIB1 that are phosphorylated by MAPK, seven potential MAPK phosphorylation sites were targeted for deletion and mutations. In vitro phosphorylation of the point mutations and internal deletions by Erk2 revealed 4 major sites of phosphorylation. In the future we plan to test these mutants for their ability to function as coactivators. Our study will help to understand how AIB1 activity is modulated by MAPK. This may help to determine how AIB1 is involved in the development of breast cancer.					
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Introduction

Our laboratory has been interested in unraveling the cellular and molecular mechanisms underlying estrogen responses in both normal and neoplastic tissues. Estrogens have central roles in the control of development, sexual behavior and reproductive functions. Their effects have also been linked to the progression of a majority of human breast cancers. The diverse biological effects of estrogens are mediated by the estrogen receptors (ER), which are members of the nuclear receptor (NR) superfamily¹. ER also associates with different classes of coregulatory factors that have either coactivator or corepressor function. The coactivators potentiate transcriptional activation by ER in an agonist-, but not antagonist-dependent manner. In general, these coactivators can be divided into five families: (1) the p160 family of proteins, including SRC-1/ERAP160/NCoA-1^{2,3}, TIF2/GRIP1/NCoA-2⁴⁻⁶, and p/CIP/ACTR/AIB1/RAC3⁷⁻¹⁰; (2) the non-p160 members, such as ARA, RIP140, TIF1 and Trip/SUG1¹¹⁻¹⁴; (3) the cointegrator CBP/p300 and its associating protein p/CAF^{15,16}; (4) the distinct DRIP/TRAP/ARC complex¹⁷⁻¹⁹; and (5) the ATP-binding regulators, such as hBrm and BRG-1^{20,21}. With identification of these coactivators, the central question by which mechanisms these multiple factors regulate ER activity remains.

Using a biochemical approach, our laboratory has identified two human ER coactivators, ERAP160 (independently identified as SRC-1 and NCoA-1) and ERAP140³. Although both proteins interact with the ER hormone binding domain (HBD) in an agonist-dependent manner, and both enhance transcriptional activation by ER, they share little sequence and structural similarities.

AIB1 (Amplified in Breast Cancer) was discovered as a gene that is amplified in certain breast and ovarian cancers^{9,10}, and our lab recently identified AIB1 as a target of the MAPK signaling pathway²². This signaling pathway is triggered, for example, by growth factors of the insulin-like growth factor (IGF) and epidermal growth factor (EGF) family. These growth factors and their receptors have also been implicated in the development and progression of breast tumors. We propose that the phosphorylation of AIB1 by MAPK may represent part of the molecular mechanism that integrates signals from steroid hormones and growth factors. Furthermore, we hypothesize that AIB1 phosphorylation may contribute to the role that AIB1 plays in the development of breast cancer.

The first goals for this proposal are to identify the sites in AIB1 that are phosphorylated by MAPK *in vitro* and *in vivo* and to determine the importance of these phosphorylation sites for the function of AIB1 as a transcriptional activator.

Our study will help to understand how AIB1 is modulated by MAPK. This may help to determine how AIB1 may be involved in the development of breast cancer, which is the most common cancer among women and the second leading cause of cancer deaths among women in U.S.A.

Body

An 864 amino acid domain of AIB1 responds maximally to MAPK *in vivo*. As mentioned in the introduction, our lab previously mapped the region of AIB1 that is functionally responsive to MAPK. Using transient transfection assays using several AIB1 deletion mutants expressed as GAL4 fusion proteins, the highest response to MAPK signaling was obtained with the mutant GAL4-AIB1 (aa 556-1420), further on called fragment 12. This mutant contains a regulatory domain responsible for ER interaction and activation domains AD1 and AD2 (Fig.1).

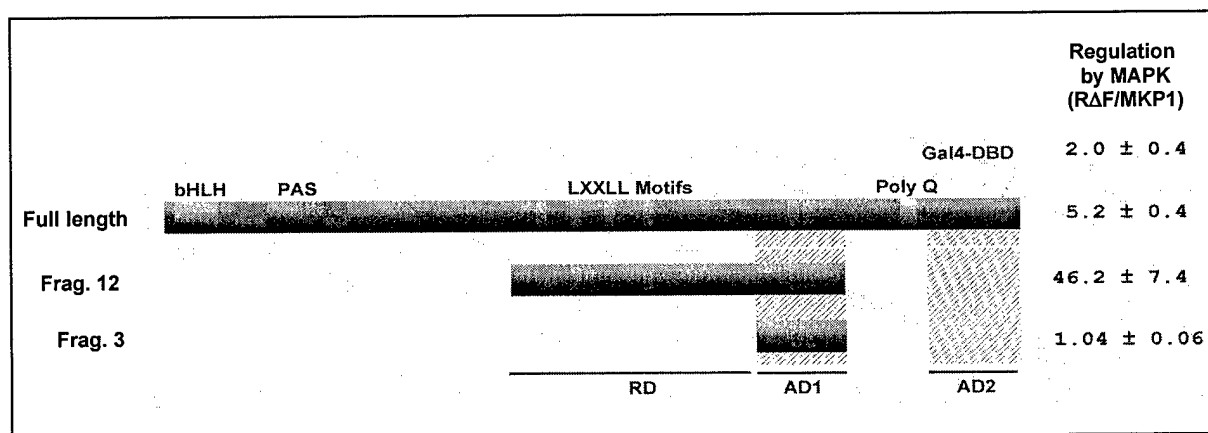


Figure 1. AIB1 Structure and regulation by MAPK. Fragment 12 showed maximum induction in transient transfection of COS cells.

***In vitro* phosphorylation of AIB1 by MAPK is restricted to the AIB1 regulatory domain.** To investigate whether AIB1 is a direct target of MAPK phosphorylation, or whether the observed functional effect of MAPK on AIB1 occurs in an indirect manner, two AIB1 fragments were used for an *in vitro* phosphorylation assay. The two fragments, fragment 12 (aa 556-1420) and fragment 3 (aa 980-1131) were generated as GST fusion proteins, in the vector pGEX-4T-1. These deletion mutants were sequenced to ensure that no mutations or undesired deletions were introduced. The proteins were expressed in *E.coli*, bound to GST beads and MAPK assays were performed using [γ - 32 P] ATP and the MAPK family member Erk2 as kinase. Myelin B protein was used as positive control and GST beads alone as negative control. Bands were analyzed by SDS gel electrophoresis and autoradiography. The experiments were carried out 4 times with similar results.

Fragment 12 was strongly phosphorylated, almost as strong as the positive control (Fig. 2a). In contrast, fragment 3 showed very little phosphorylation even though fragment 3 showed better expression in the loading control (Fig. 2b). These results indicate that AIB1 phosphorylation by MAPK is restricted to the regulatory domain within fragment 12.

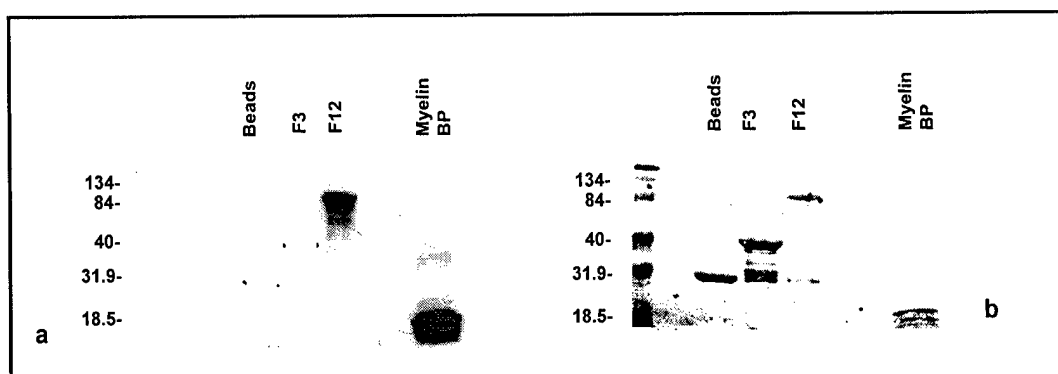


Figure 2. AIB1 phosphorylation by Erk2. a) Autoradiography of AIB1 *in vitro* phosphorylation. Fragment 3 and 12 were generated as GST fusion proteins. *In vitro* phosphorylation was performed using Erk2 as kinase. b) Coomassie stain of SDS-PAGE gel from a) as loading control.

Precise mapping of AIB1 phosphorylation sites *in vitro*.

Based on these results the amino acid sequence of fragment 12 was examined for the presence of consensus phosphorylation sites for MAP kinase (PXX(S/T)P) and 7 potential phosphorylation sites termed a-g, see Fig 3) were found. Of note, some of these sites but not all are conserved in Src1 and/or TIF2^{23,24} (see Fig. 3). The seven sites were targeted by site directed mutagenesis to determine the contribution of these sites for the whole phosphorylation pattern of AIB1 by MAPK. All point mutations were generated as Serine to Alanine mutations in the pGEX-4T-1 vector using a Stratagene mutagenesis kit. In addition to the point mutations, three internal deletions were generated to eliminate several phosphorylation sites at once (Fig.3).

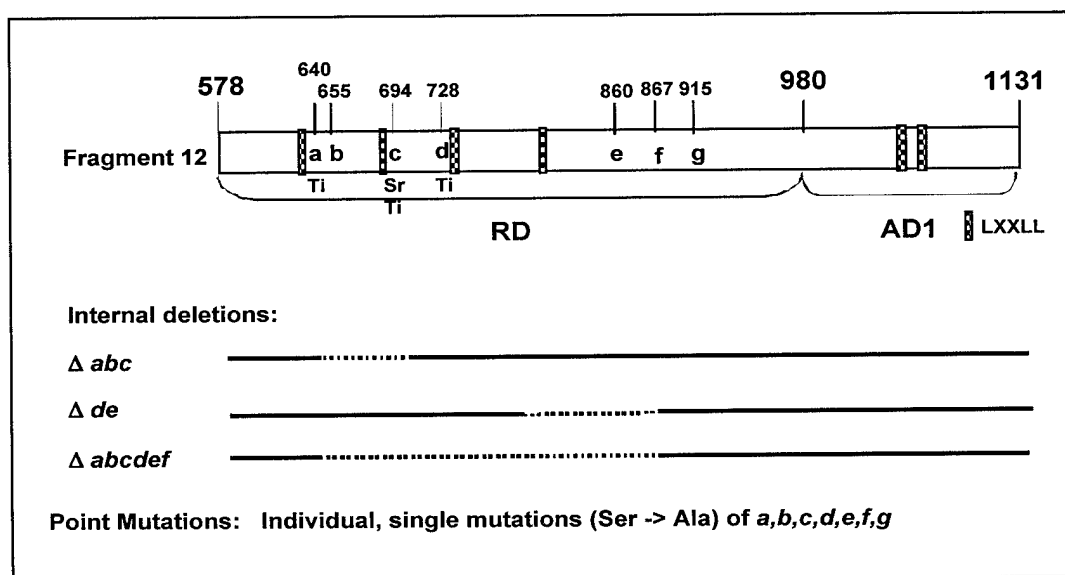


Figure 3. Mutational analysis of potential MAPK sites in AIB1 regulatory domain

After sequencing all the mutants in order to ensure that no undesired mutations were introduced, protein from all mutants was produced in *E.coli*. Once the protein products were bound to GST beads *in vitro* phosphorylation assays were carried out using Erk2

as kinase (Fig. 4). The deletion or mutation of sites **a**, **b**, **c** did not result in a significant change of signal intensity, as compared to the phosphorylation of wt AIB1 fragment 12 (Fig. 4). In sharp contrast, deletion of sites **d**, **e**, **f** or mutation of sites **e** and **d** showed strongly reduced phosphorylation. Site **e** seems to be the preferred site for phosphorylation by MAPK, since less protein was loaded as compared to **d** as seen in the Coomassie staining (Fig. 4 lower panel). Altogether, we conclude that MAPK phosphorylates AIB 1 predominantly on sites **d**, **e**, **f**, or **g**, while **a**, **b**, **c** are clearly far less involved in phosphorylation.

As a next step, we will investigate the relevance of all of these phosphorylation sites for the coactivator function of AIB1 *in vivo*. We expect to be able to correlate *in vitro* phosphorylation by MAPK with *in vivo* responsiveness of AIB1 to MAPK. This would represent the definitive proof that the modulation of AIB1 function by MAPK is the result of direct phosphorylation of AIB1 by MAPK.

All of the phosphorylation mutants will be generated as GAL4 fusion constructs and examined in a transient transfection assay for their ability to respond to MAPK, in analogy to previously published experiments²².

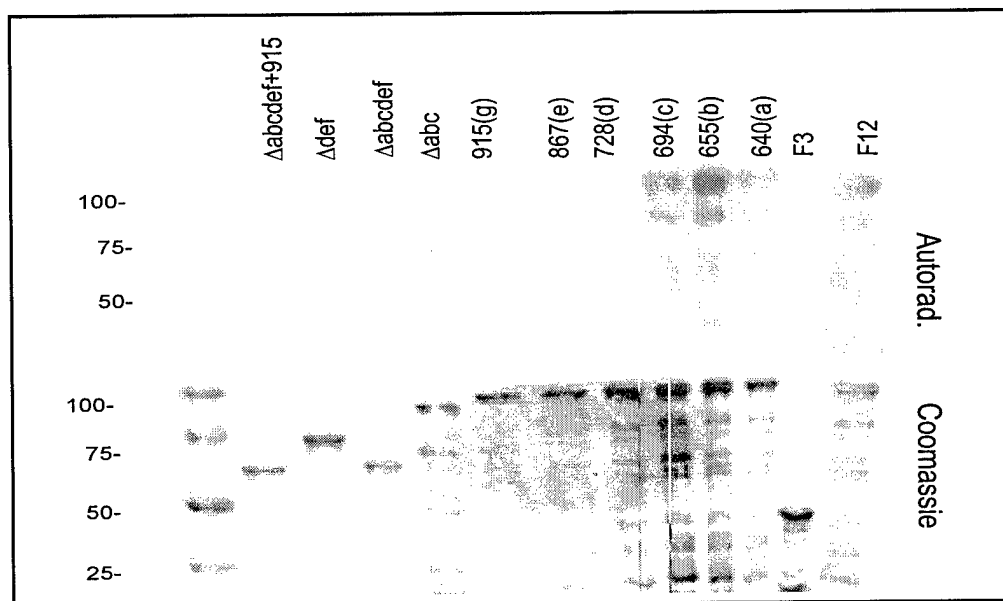


Figure 4. In vitro phosphorylation of AIB1 internal deletions and point mutations by MAPK (Erk2). Upper panel autoradiography and lower panel Coomassie staining of the same SDS-PAGE gel.

Key Research Accomplishments

- The regions of AIB1 phosphorylated by MAPK *in vitro* were mapped, using deletion mutants of AIB1.
- Phosphorylation of AIB1 by MAPK *in vitro* was found to be restricted to the AIB1 regulatory domain.
- The precise amino acids within the AIB1 regulatory domain that are phosphorylated by MAPK *in vitro* were identified.

Conclusions

We found that most of the AIB1 phosphorylation by MAPK occurs within the regulatory domain of AIB1. This result is consistent with our previous data, indicating that this domain is also required for the maximal induction of AIB1 coactivator function by MAPK, *in vivo*. Furthermore, our analysis of point mutations of the 7 putative MAPK consensus sites in this region indicates that the *in vitro* phosphorylation of AIB1 fragment 12 by MAPK is a specific effect that occurs only at a subset of these sites. A functional analysis of these mutations as GAL4 fusion constructs *in vivo* in cell lines should be able to confirm the biological significance of these results and establish AIB1 as a target of growth factor signaling. Together with the coactivator function of AIB1, this would represent further evidence of a causative role of AIB1 in breast cancer.

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